Dkt. 0575/62097/JPW/JML

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that **David M. Stern, Ann Marie Schmidt, Shi Du Yan, and Berislav Zlokovic** have invented certain new and useful improvements in

A Method To Increase Cerebral Blood Flow In Amyloid Angiopathy

of which the following is a full, clear and exact description.

Dkt. 0575/62097/JPW/JML

A Method to Increase Cerebral Blood Flow In Amyloid Angiopathy

The invention disclosed herein was made with Government support under Grant No. P01AG16233 from the National Institutes of Health of the U.S. Department of Public Health. Accordingly, the U.S. Government has certain rights in this invention.

Background of the Invention

Throughout this application, various publications are referenced by number. Full citations for these publications may be found listed at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The pain of Alzheimer's disease results directly from the memory loss and cognitive deficits suffered by the patient. These eventually result in the patient's loss of identity, autonomy, and freedom. As a step toward curing this disease, alleviating its symptoms, or retarding its progression, it would be desirable to develop a transgenic animal model exhibiting the main debilitating phenotype of Alzheimer's disease, that is, memory loss, expressed concomitantly with the neuropathological correlates of Alzheimer's disease, for example, beta-amyloid accumulation, increased glial reactivity, and hippocampal cell loss.

It is estimated that over 5% of the U.S. population over 65 and over 15% of the U.S. population over 85 are beset with some form of Alzheimer's disease (Cross, A. J., Eur J Pharmacol (1982) 82:77-80; Terry, R. D., et al., Ann Neurol (1983) 14:497506). It is believed that the principal cause for confinement of the elderly in long term care facilities is due to this disease, and approximately 65% of those dying in skilled nursing facilities suffer from it.

Certain facts about the biochemical and metabolic phenomena associated with the presence of Alzheimer's disease are known. Two morphological and histopathological changes noted in Alzheimer's disease brains are neurofibrillary tangles (NFT) and amyloid deposits. Intraneuronal neurofibrillary tangles are present in other degenerative diseases as well, but the presence of amyloid deposits both in the interneuronal spaces (neuritic plaques) and in the surrounding microvasculature (vascular plaques) seems to be characteristic of Alzheimer's. Of these, the neuritic plaques seem to be the most prevalent (Price, D. L., et al., Drug Development Research (1985) 5:59-68). Plaques are also seen in the brains of aged Down's Syndrome patients who develop Alzheimer's disease.

Summary of the Invention

The present invention provides a method for decreasing cerebral vasoconstriction in a subject suffering from chronic or acute cerebral amyloid angiopathy which comprises administering to the subject an inhibitor of receptor for advanced glycation endproduct (RAGE) in an effective amount to inhibit transcytosis of amyloid β peptides across the blood-brain barrier in the subject, thereby decreasing cerebral vasoconstriction in the subject. The invention further provides for a method for ameliorating neurovascular stress in a subject which comprises administering to the subject an effective amount of an inhibitor of receptor for advanced glycation endproduct (RAGE), so as to increase cerebral blood flow in the subject, thereby ameliorating neurovascular stress in the subject.

Brief Description of the Figures

Figures. 1A-1F. RAGE-dependent Amyloid beta (Aβ) binding to brain endothelium and *in vivo* transcytosis across the blood brain barrier (BBB) followed by rapid neuronal uptake of circulating Aβ in mice. ***Figure 1A*** and ***bFigure 1B,*** Binding to brain capillaries (*a*) and transport across the BBB (uptake by capillary-depleted brain expressed as cerebrovascular permeability product, PS) (*b*) of ^{125}I-labeled human $A\beta_{1-40}$ ($hA\beta_{1-40}{}^*$) and $A\beta_{1-42}$ ($hA\beta_{1-42}{}^*$), and murine $A\beta_{1-40}$ ($mA\beta_{1-40}{}^*$) infused into cerebral arterial circulation at 4 nM for 10 min via brain perfusion technique in the absence and presence of α-RAGE (40 mg/ml), sRAGE (40 nM), SR, scavenger receptor ligand - fucoidan (100 mg/ml), FNR5 (anti-β1-integrin antibody, 40 mg/ml) or RHDS (40 nM); $hA\beta_{40-1}{}^*$ denotes ^{125}I-labeled scrambled peptide. ***Figure 1C*** and ***Figure 1D,*** Dose-dependent effect of α-RAGE (0.5 to 40 mg/ml) on brain capillary binding (*c*) and transport across the BBB (*d*) of ^{125}I-$A\beta_{1-40}$ ($hA\beta_{1-40}{}^*$). ***Figure 1E,*** Partial metabolic degradation of human Abβ1-40 ($hA\beta_{1-40}{}^*$) and $A\beta_{1-42}$ ($hA\beta_{1-42}{}^*$) in brain parenchyma following 10 min of BBB transport of circulating ^{125}I-labeled peptides. ***Figure 1F,*** Immunocytochemical detection of $hA\beta_{1-40}$ with anti-$A\beta_{1-40}$ antibody (QCB) in brain parenchyma 10 min after its BBB transport in the absence (middle panel) and presence of α-RAGE, 40 mg/ml (right panel); control vehicle-infused brain is shown on a right panel. n = 3 to 5 mice per group. $^*p < 0.01$.

Figures 2A-2D. Effect of RAGE blockade on Aβ-induced cytokine expression and oxidant stress in brain after BBB transport of circulating $A\beta_{1-40}$. Expression of TNF-α mRNA (*left*) and protein (*right*) (***Figure 2A***), and immunocytochemical detection of IL-6 (***Figure 2B***) and HO-1

(***Figure 2C***) 15 min following transport of human $A\beta_{1-40}$ (4 nM) across the BBB in the presence or absence of α-RAGE (40 mg/ml) or sRAGE (40 nM) in the arterial inflow in a brain perfusion model. Vehicle-infused brains were also shown in *Figures 2A-2C* as control. Graphs in *Figures 2A-2C* illustrate image analysis of immunocytochemical experiments in which mice were treated with either vehicle, $A\beta_{1-40}$ alone, or $A\beta_{1-40}$ plus α-RAGE or sRAGE, as indicated. ***Figure 2D***, Image analysis of mouse brains after 2 hrs of i.v. administration of $A\beta_{1-40}$ (4 nM) in the absence and presence of α-RAGE (40 mg/ml) or sRAGE (40 nM) infused 15 min prior to $A\beta_{1-40}$ infusion. n = 5 mice per group. $^*p < 0.01$.

Figures 3A-3C. RAGE-dependent vasomotor effects of circulating $A\beta$. Decrease in CBF following i.v. administration of human $A\beta_{1-40}$ (4 nM) (***Figure 3A***) and effect of α-RAGE (40 mg/ml) (***Figures 3B-C***). α-RAGE (1-10 mg/ml) and sRAGE (40 nM) blocked CBF changes produced by murine or human $A\beta_{1-40}$; CBF values between 30 and 45 min after i.v. administration of peptides. sRAGE (40 nM) and IgG, lack of effect of an irrelevant IgG. n = 5 mice per group; $^*p < 0.01$.

Figures 4A-4D. Effects of RAGE blockade on cerebral blood flow (CBF) in TgAPPsw+/- mice. ***Figure 4A,*** Baseline CBF values and arterial blood pressure in 9 months old TgAPPsw+/- mice and aged-matched control mice. ***Figure 4B,*** Significant increase in CBF in 9 months old TgAPPsw+/- mice following administration of a-RAGE (40 mg/ml); IgG, non-specific immunoglobulin ***Figure 4C,*** Image analysis of brains in TgAPPsw+/- mice for TNF-a, IL-6 and HO-1 2 hrs following treatment with either vehicle or α-RAGE (40 mg/ml). ***Figure 4D,*** Increased vascular expression of RAGE and $A\beta$ accumulation in Alzheimer's Disease (AD) brain. n = 5 mice per group; $^*p$

< 0.01.

Detailed Description of the Invention

This invention provides for a method for decreasing cerebral vasoconstriction in a subject suffering from chronic or acute cerebral amyloid angiopathy which comprises administering to the subject an inhibitor of receptor for advanced glycation endproduct (RAGE) in an effective amount to inhibit transcytosis of amyloid β peptides across the blood-brain barrier in the subject, thereby decreasing cerebral vasoconstriction in the subject.

In one embodiment of the invention, the subject is a transgenic non-human animal or a human. In another embodiment of the invention, the non-human animal is a transgenic mouse which over-expresses mutant human amyloid beta precursor protein. In another embodiment of the invention, the subject suffers from Alzheimer's disease. In another embodiment of the invention, the chronic cerebral amyloid angiopathy is due to Alzheimer's disease, Down's syndrome, aging or angiopathy. In another embodiment of the invention, the acute cerebral amyloid angiopathy is due to head trauma, or stroke.

In one embodiment of the invention, the inhibitor is a molecule having a molecular weight from about 500 daltons to about 100 kilodaltons. In another embodiment of the invention, the inhibitor is an organic molecule or an inorganic molecule. In another embodiment of the invention, the inhibitor is a polypeptide or a nucleic acid molecule. In another embodiment of the invention, the inhibitor is soluble receptor for advanced glycation endproduct. In another embodiment of the invention, the inhibitor is an antibody which specifically binds to receptor for advanced

glycation endproduct.

The invention also provides for a method for ameliorating neurovascular stress in a subject which comprises administering to the subject an effective amount of an inhibitor of receptor for advanced glycation endproduct (RAGE), so as to increase cerebral blood flow in the subject, thereby ameliorating neurovascular stress in the subject.

In one embodiment of the invention, the inhibitor of receptor for advanced glycation endproduct (RAGE) is soluble receptor for advanced glycation endproduct (RAGE). In another embodiment of the invention, the neurovascular stress comprises cerebral amyloid angiopathy. In another embodiment of the invention, the neurovascular stress in the subject is caused by Alzheimer's disease, aging, Down's syndrome, head trauma, or stroke.

The invention also provides for a method for treating amyloid angiopathy in a subject who suffers therefrom which comprises administering to the subject an effective amount of an inhibitor of receptor for advanced glycation endproduct (RAGE) activity so as to increase cerebral blood flow in the subject and thereby treat amyloid angiopathy in the subject.

The present invention provides for a method for determining whether a compound increases cerebral blood flow in a subject which comprises: (a) administering the compound to a non-human animal which exhibits at least one of the following characteristics: a correlative memory deficit, elevation of amyloid β in the brain of the non-human animal, or amyloid β plaques in the brain of the non-human animal; (b) determining whether the non-human animal has increased

cerebral blood flow when compared to cerebral blood flow in an identical non-human animal which was not administered the test compound; wherein an increase in cerebral blood flow indicates that the test compound increases cerebral blood flow in a subject.

In one embodiment of the invention, the non-human animal is a transgenic non-human animal. In another embodiment of the invention, the non-human animal is a transgenic mouse which over-expresses mutant human amyloid beta precursor protein. In another embodiment of the invention, the non-human animal is a transgenic non-human animal which is an animal model for Alzheimer's disease.

In one embodiment of the invention, the non-human animal is a Swiss transgenic mouse designated Tg APP sw+/-.

4.In one embodiment of the invention, the compound is a molecule having a molecular weight from about 500 daltons to about 100 kilodaltons. In one embodiment of the invention, the compound is an organic molecule or an inorganic molecule. In one embodiment of the invention, the compound is a polypeptide or a nucleic acid molecule.

The invention also provides for a method for ameliorating neurovascular stress in a subject which comprises administering to the subject an effective amount of an inhibitor of RAGE, so as to increase cerebral blood flow in the subject, thereby ameliorating neurovascular stress in the subject.

In one embodiment of the invention, the inhibitor of RAGE

is soluble RAGE. In another embodiment of the invention, the neurovascular stress comprises amyloid angiopathy. In another embodiment of the invention, the neurovascular stress is caused by Alzheimer's disease or aging of the subject.

The invention also provides for a method for treating amyloid angiopathy in a subject who suffers therefrom which comprises administering to the subject an effective amount of an inhibitor of receptor for advanced glycation endproduct (RAGE) activity so as to increase cerebral blood flow in the subject and thereby treat amyloid angiopathy in the subject.

The invention also provides for a method for treating cerebral amyloid angiopathy in a subject who suffers therefrom which comprises administering to the subject an effective amount of a compound determined to inhibit activity of receptor for advanced glycation endproducts (RAGE) in the method described hereinabove for determining whether a compound increases cerebral blood flow in a subject.

Definitions

"DNA sequence" is a linear sequence comprised of any combination of the four DNA monomers, i.e., nucleotides of adenine, guanine, cytosine and thymine, which codes for genetic information, such as a code for an amino acid, a promoter, a control or a gene product. A specific DNA sequence is one which has a known specific function, e.g., codes for a particular polypeptide, a particular genetic trait or affects the expression of a particular phenotype.

"Genotype" is the genetic constitution of an organism.

"Phenotype" is a collection of morphological, physiological and biochemical traits possessed by a cell or organism that results from the interaction of the genotype and the environment.

"Phenotypic expression" is the expression of the code of a DNA sequence or sequences which results in the production of a product, e.g., a polypeptide or protein, or alters the expression of the zygote's or the organisms natural phenotype.

"Zygote" is a diploid cell having the potential for development into a complete organism. The zygote can result from parthenogenesis, nuclear transplantation, the merger of two gametes by artificial or natural fertilization or any other method which creates a diploid cell having the potential for development into a complete organism. The origin of the zygote can be from either the plant or animal kingdom.

In the practice of any of the methods of the invention or preparation of any of the pharmaceutical compositions an "therapeutically effective amount" is an amount which is capable of alleviating the symptoms of the disorder of memory or learning in the subject. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated. For the purposes of this invention, the methods of administration are to include, but are not limited to, administration cutaneously, subcutaneously, intravenously, parenterally, orally, topically, or by aerosol.

By "nervous system-specific" is meant that expression of a

nucleic acid sequence occurs substantially in a nervous system tissue (for example, the brain or spinal cord). Preferably, the expression of the nucleic acid sequence in the nervous system tissue represents at least a 5-fold, more preferably, a 10-fold, and, most preferably, a 100-fold increase over expression in non-nervous system tissue.

The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, sheep, dog, cow, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably mouse.

The "transgenic non-human animals" of the invention are produced by introducing "transgenes" into the germline of the non-human animal.

__Nucleotide and Amino Acid sequences of RAGE__

The nucleotide and protein (amino acid) sequences for RAGE (both human and murine and bovine) are known. The following references which recite these sequences are incorporated by reference:

Schmidt et al, J. Biol. Chem., 267:14987-97, 1992
Neeper et al, J. Biol. Chem., 267:14998-15004, 1992

RAGE sequences (DNA sequence and translation) from bovine, murine and homo sapien are listed hereinbelow. These sequences are available from GenBank as are other sequences of RAGE from other species:

LOCUS BOVRAGE 1426 bp mRNA MAM 09-DEC-1993 DEFINITION Cow

receptor for advanced glycosylation end products (RAGE) mRNA, complete cds.

ACCESSION M91212VERSION M91212.1 GI:163650

KEYWORDS RAGE; cell surface receptor.

SOURCE Bos taurus cDNA to mRNA. ORGANISM Bos taurus Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Cetartiodactyla; Ruminantia; Pecora; Bovoidea; Bovidae; Bovinae; Bos.

REFERENCE 1 (bases 1 to 1426) AUTHORS Neeper,M., Schmidt,A.M., Brett,J., Yan,S.D., Wang,F., Pan,Y.C., Elliston,K., Stern,D. and Shaw,A. TITLE Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins

JOURNAL J. Biol. Chem. 267, 14998-15004 (1992)

MEDLINE 92340547 REFERENCE 2 (bases 1 to 1426) AUTHORS Shaw,A. TITLE Direct Submission JOURNAL Submitted (15-APR-1992) A. Shaw, Department of Cellular and Molecular Biology, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486

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LOCUS HUMRAGE 1391 bp mRNA PRI 09-DEC-1993

DEFINITION Human receptor for advanced glycosylation end products (RAGE) mRNA, partial cds.

ACCESSION M91211VERSION M91211.1 GI:190845

KEYWORDS RAGE; cell surface receptor.

SOURCE Homo sapiens cDNA to mRNA.

ORGANISM Homo sapiens Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 1391)

AUTHORS Neeper,M., Schmidt,A.M., Brett,J., Yan,S.D., Wang,F., Pan,Y.C., Elliston,K., Stern,D. and Shaw,A.

TITLE Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins

JOURNAL J. Biol. Chem. 267, 14998-15004 (1992)

MEDLINE 92340547

REFERENCE 2 (bases 1 to 1391)

AUTHORS Shaw,A.

TITLE Direct Submission

JOURNAL Submitted (15-APR-1992) A. Shaw, Department of Cellular and Molecular Biology, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486 USA

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LOCUS MUSRECEP 1348 bp mRNA ROD 23-AUG-1994

DEFINITION Mouse receptor for advanced glycosylation end products (RAGE) gene, complete cds.

ACCESSION L33412VERSION L33412.1 GI:532208

KEYWORDS receptor for advanced glycosylation end products.

SOURCE Mus musculus (strain BALB/c, sub_species domesticus) (library: lambda gt10) male adult lung cDNA to mRNA.

ORGANISM Mus musculus Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.

REFERENCE 1 (bases 1 to 1348)

AUTHORS Lundh,E.R., Morser,J., McClary,J. and Nagashima,M.

TITLE Isolation and characterization of cDNA encoding the murine and rat homologues of the mammalian receptor for advanced glycosylation end products

JOURNAL UnpublishedCOMMENT On Aug 24, 1994 this sequence version replaced gi:496146.

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901 gctgcgtggc cacccaccct agccacggac ctcaggaaag ccctcctgtc agcatcaggg
961 tcacagaaac cggcgatgag gggccagctg aaggctctgt gggtgagtct gggctgggta
1021 cgctagccct ggccttgggg atcctgggag gcctgggagt agtagccctg ctcgtcgggg
1081 ctatcctgtg gcgaaaacga caacccaggc gtgaggagag gaaggccccg gaaagccagg
1141 aggatgagga ggaacgtgca gagctgaatc agtcagagga agcggagatg ccagagaatg
1201 gtgccggggg accgtaagag cacccagatc gagcctgtgt gatggcccta gagcagctcc
1261 cccacattcc atcccaattc ctccttgagg cacttccttc tccaaccaga gcccacatga
1321 tccatgctga gtaaacattt gatacggc// **(SEQ ID NO:6)**

Inhibitors of RAGE:

Inhibitors of RAGE include any molecule which, when introduced into a cell or a subject, is capable of inhibiting the biological activity of RAGE. For example, one such inhibitor would be able to inhibit the activity of RAGE as described: the activity of transcytosis of amyloid beta peptides across the blood brain barrier within a subject.

Examples of an inhibitor of RAGE activity are soluble RAGE, an antibody which specifically binds to RAGE, a truncated version of RAGE which is capable of acting as a competitive inhibitor of RAGE. A fragment of RAGE which includes the amyloid beta peptide binding portion of RAGE and introduced into the cell or subject as a soluble polypeptide. Other types of inhibitors would be known to one of skill in the art. For example, a small molecule could be prepared which mimics the amyloid beta peptide binding region of RAGE and administered alone as an inhibitor.

Pharmaceutical compositions and Carriers

As used herein, the term "suitable pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

This invention also provides for pharmaceutical compositions including therapeutically effective amounts of protein compositions and compounds together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful in treatment of neuronal degradation due to aging, a learning disability, or a neurological disorder. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the compound, complexation with metal ions, or incorporation of the compound into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, micro emulsions, micelles, unilamellar or multi lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the compound or composition. The choice of compositions will depend on the physical and chemical properties of the compound.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

Portions of the compound of the invention may be "labeled" by association with a detectable marker substance (e.g., radiolabeled with ^{125}I or biotinylated) to provide reagents useful in detection and quantification of compound or its receptor bearing cells or its derivatives in solid tissue and fluid samples such as blood, cerebral spinal fluid or urine.

When administered, compounds are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive compounds may by required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in

aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less frequently or in lower doses than with the unmodified compound.

Attachment of polyethylene glycol (PEG) to compounds is particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species without the risk of triggering a severe immune response. The compound of the present invention capable of alleviating symptoms of a cognitive disorder of memory or learning may be delivered in a microencapsulation device so as to reduce or prevent an host immune response against the compound or against cells which may produce the compound. The compound of the present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the amino terminal amino acid, the epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group

of the carboxy-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

In one embodiment the compound of the present invention is associated with a pharmaceutical carrier which includes a pharmaceutical composition. The pharmaceutical carrier may be a liquid and the pharmaceutical composition would be in the form of a solution. In another embodiment, the pharmaceutically acceptable carrier is a solid and the composition is in the form of a powder or tablet. In a further embodiment, the pharmaceutical carrier is a gel and the composition is in the form of a suppository or cream. In a further embodiment the active ingredient may be formulated as a part of a pharmaceutically acceptable transdermal patch.

Transgenic Technology and Methods

The following U.S. Patents are hereby incorporated by

reference: U.S. Patent No. 6,025,539, IL-5 transgenic mouse; U.S. Patent No. 6,023,010, Transgenic non-human animals depleted in a mature lymphocytic cell-type; U.S. Patent No. 6,018,098, In vivo and in vitro model of cutaneous photoaging; U.S. Patent No. 6,018,097, Transgenic mice expressing human insulin; U.S. Patent No. 6,008,434, Growth differentiation factor-11 transgenic mice; U.S. Patent No. 6,002,066; H2-M modified transgenic mice; U.S. Patent No. 5,994,618, Growth differentiation factor-8 transgenic mice; U.S. Patent No. 5,986,171, Method for examining neurovirulence of polio virus, U.S. Patent No. 5,981,830, Knockout mice and their progeny with a disrupted hepsin gene; U.S. Patent No. 5,981,829, .DELTA.Nur77 transgenic mouse; U.S. Patent No. 5,936,138; Gene encoding mutant L3T4 protein which facilitates HIV infection and transgenic mouse expressing such protein; U.S. Patent No. 5,912,411, Mice transgenic for a tetracycline-inducible transcriptional activator; U.S. Patent No. 5,894,078, Transgenic mouse expressing C-100 app.

The methods used for generating transgenic mice are well known to one of skill in the art. For example, one may use the manual entitled "Manipulating the Mouse Embryo" by Brigid Hogan et al. (Ed. Cold Spring Harbor Laboratory) 1986.

See for example, Leder and Stewart, U.S. Patent No. 4,736,866 for methods for the production of a transgenic mouse.

For sometime it has been known that it is possible to carry out the genetic transformation of a zygote (and the embryo and mature organism which result therefrom) by the placing or insertion of exogenous genetic material into the nucleus of the zygote or to any nucleic genetic material which

ultimately forms a part of the nucleus of the zygote. The genotype of the zygote and the organism which results from a zygote will include the genotype of the exogenous genetic material. Additionally, the inclusion of exogenous genetic material in the zygote will result in a phenotype expression of the exogenous genetic material.

The genotype of the exogenous genetic material is expressed upon the cellular division of the zygote. However, the phenotype expression, e.g., the production of a protein product or products of the exogenous genetic material, or alterations of the zygote's or organism's natural phenotype, will occur at that point of the zygote's or organism's development during which the particular exogenous genetic material is active. Alterations of the expression of the phenotype include an enhancement or diminution in the expression of a phenotype or an alteration in the promotion and/or control of a phenotype, including the addition of a new promoter and/or controller or supplementation of an existing promoter and/or controller of the phenotype.

The genetic transformation of various types of organisms is disclosed and described in detail in U.S. Pat. No. 4,873,191, issued Oct. 10, 1989, which is incorporated herein by reference to disclose methods of producing transgenic organisms. The genetic transformation of organisms can be used as an in vivo analysis of gene expression during differentiation and in the elimination or diminution of genetic diseases by either gene therapy or by using a transgenic non-human mammal as a model system of a human disease. This model system can be used to test putative drugs for their potential therapeutic value in humans.

The exogenous genetic material can be placed in the nucleus of a mature egg. It is preferred that the egg be in a fertilized or activated (by parthenogenesis) state. After the addition of the exogenous genetic material, a complementary haploid set of chromosomes (e.g., a sperm cell or polar body) is added to enable the formation of a zygote. The zygote is allowed to develop into an organism such as by implanting it in a pseudopregnant female. The resulting organism is analyzed for the integration of the exogenous genetic material. If positive integration is determined, the organism can be used for the in vivo analysis of the gene expression, which expression is believed to be related to a particular genetic disease.

Attempts have been made to study a number of different types of genetic diseases utilizing such transgenic animals. Attempts related to studying Alzheimer's disease are disclosed within published PCT application WO89/06689 and PCT application WO89/06693, both published on Jul. 27, 1989, which published applications are incorporated herein by reference to disclose genetic sequences coding for Alzheimer's .beta.-amyloid protein and the incorporation of such sequences into the genome of transgenic animals.

Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2 pl of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated

into the host gene before the first cleavage (Brinster, et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4438-4442). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Microinjection of zygotes is the preferred method for incorporating transgenes in practicing the invention.

Retroviral infection can also be used to introduce transgene into a non-human animal. The developing non-human embryo can be cultured in vitro to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, R. (1976) Proc. Natl. Acad. Sci U.S.A. 73, 1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan, et al. (1986) in Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner, et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 6927-6931; Van der Putten, et al. (1985) Proc. Natl. Acad. Sci U.S.A. 82, 6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, supra; Stewart, et al. (1987) EMBO J. 6, 383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner, D., et al. (1982) Nature 298, 623-628). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various

retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner, D. et al. (1982) supra).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured in vitro and fused with embryos (Evans, M. J., et al. (1981) Nature 292, 154-156; Bradley, M. O., et al. (1984) Nature 309, 255-258; Gossler, et al. (1986) Proc. Natl. Acad. Sci U.S.A. 83, 9065-9069; and Robertson, et al. (1986) Nature 322, 445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) Science 240, 1468-1474.

As used herein, a "transgene" is a DNA sequence introduced into the germline of a non-human animal by way of human intervention such as by way of the above described methods.

The disclosures of publications referenced in this application in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Example 1: Receptor for Advanced Glycation Endproduct (RAGE)-dependent neurovascular dysfunction caused by amyloid-ß peptide

Amyloid-beta peptides (Aβ) are important in the pathogenesis of Alzheimer's dementia. We show that RAGE mediates Aβ transport across the blood-brain barrier (BBB) in mice followed by its rapid neuronal uptake, cytokine response, oxidant stress and reductions in the cerebral blood flow (CBF). Antagonizing RAGE in transgenic mice that overexpress mutant human Aβ precursor protein restored the CBF and ameliorated neurovascular stress. In Alzheimer's brains, vascular expression of RAGE was associated with Aβ accumulation. We suggest that RAGE at the BBB is a potential target for inhibiting vascular accumulation of Aβ and for limiting cellular stress and ischemic changes in Alzheimer's dementia.

Deposition of Aß in the CNS occurs during normal aging and is accelerated by Alzheimer's Disease (AD). [1-4] Aß is implicated in neuropathology of AD and related disorders. [1-4] Aß peptides have neurotoxic properties *in vitro* [5-7] and *in vivo*, [8-10] and induce neuronal oxidant stress directly and indirectly by activating microglia. [11-13] Aß generates superoxide radicals in brain endothelium, [14] and at higher concentrations may damage endothelial cells. [15] Recent studies from our and other laboratories suggest a major role of the blood-brain barrier (BBB) in determining the concentrations of Aß in the CNS. [16-25] The BBB controls the entry of plasma-derived Aß and its binding transport proteins into the CNS, and regulates the levels of brain-derived Aß

via clearance mechanisms.

RAGE (receptor for advanced glycation end-product), a multiligand receptor in the immunoglobulin superfamily binds free Aß in the nanomolar range, and mediates pathophysiological cellular responses when occupied by glycated ligands, Aß, S100/calgranulins or serum amyloid A.[24,26-28] RAGE is up-regulated on microglia and vascular endothelium in AD brains. [29,30] We have recently reported that RAGE may be involved in transport of Aß across human brain endothelial monolayers. [24,31] Our current study demonstrates that RAGE mediates *in vivo* transcytosis of Aß1-40 and Aß1-42 across the BBB in mice. RAGE-dependent BBB transport of Aß was coupled to its rapid neuronal uptake, induction of cellular stress and transient, but significant suppression of cerebral blood flow (CBF). Antagonizing RAGE in transgenic mice that overexpress mutant human Aß precursor protein (APP) restored the CBF and ameliorated cellular stress. In Alzheimer's brains, vascular expression of RAGE was associated with Aß accumulation. These data support the possibility that inhibiting RAGE at the BBB may limit vascular accumulation of Aß and reduce cellular stress and ischemic changes in Alzheimer's dementia.

RAGE mediates *in vivo* transcytosis of Aß across the BBB

RAGE-dependent binding to brain microvessels (Fig. 1a) and transport across the BBB (Fig. 1b) of human and mouse $Aß_{1-40}$, and somewhat slower, but significant RAGE-dependent BBB transport of $Aß_{1-42}$ (Fig. 1b) and absence of its significant binding to microvessels (Fig. 1a) were found in mice (shown in Fig. 1) and guinea pigs. Aß transport into brain was significantly inhibited by 65% to 85% by circulating α-RAGE

IgG (5-40μg/kg) and abolished by sRAGE. Several other molecular reagents including fucoidan (a ligand for the scavenger receptor type A), anti-β1-integrin antibodies, or RHDS peptide (5-9 sequence of Aß) did not affect either BBB transport or binding of Aß (Figs. 1a and b). Although Aß peptides were partially metabolized during their transport across the BBB (i.e., $\leq$ 50% for 10 min), significant and rapid RAGE-dependent neuronal uptake of circulating Aß was observed after the BBB transport (Fig. 1e).

Circulating Aß and RAGE-dependent neurovascular stress

Transport of Aß$_{1-40}$ across the BBB was associated with an early cellular stress response that preceded changes in the CBF. The expression of TNF-α mRNA and protein on different cells in brain parenchyma, including neurons and brain endothelium was evident after 15 min of transport of circulating Aß across the BBB (Fig. 2a). Treatment with circulating sRAGE (Fig. 2a) or α-RAGE IgG abolished Aß-induced TNF-α expression. Aß transport across the BBB resulted in rapid neuronal expression of lL-6 (Fig. 2b) and HO-1 (Fig 3c), and these effects were abolished by either α-RAGE IgG (Fig. 2 b and c) or sRAGE, supporting the concept that RAGE-dependent Aß BBB transport initiates cellular stress in brain. RAGE-dependent Aß-induced cellular stress was found either after cerebral arterial or systemic intravenous administration of Aß, and persisted in brain for few hours. Fig. 2d illustrates expression of TNF-α, IL-6 and HO-1 in brain 2 hrs after i.v. administration of Aß$_{1-40}$ at low nanomolar level.

Systemic administration of Aß$_{1-40}$ (either human or murine) at low nanomolar concentrations resulted in time-dependent

decrease in the CBF, but did not affect systemic arterial blood pressure (Fig. 3a). Reductions in the CBF were detectable after 20-30 min of Aß administration, and maximal decrease in the CBF was observed between 40-60 min. CBF changes were completely antagonized by circulating α-RAGE at 40 μg/ml (Fig. 3b). Aß-induced cerebral vasospasm was antagonized by α-RAGE in a dose-dependent manner, was abolished by sRAGE, but was not affected by an irrelevant antibody (Fig. 3c).

RAGE blockade restores the CBF in Tg APP sw+/- mice

Fig. 4a shows significant decease in basal CBF values in 9 months old Tg APPsw+/- mice compared to age-matched control mice as determined by laser Doppler flowmetry, and confirmed by quantitative autoradiographic analysis. There was no difference in the arterial blood pressure between wild type and Tg APPsw+/- mice (Fig. 4a). Infusion of α-RAGE dramatically increased the CBF in Tg APPsw+/- mice (Fig. 4b), and the effect was maximal between 60-120 min after systemic administration of α-RAGE. An irrelevant IgG did not affect the CBF in Tg APPsw+/- animals (Fig. 4b). Systemic administration of α-RAGE ameliorated cellular stress in brain of 9 month old Tg APPsw+/- mice, as indicated by moderate reduction in expression of TNF-a, IL-6 and HO-1 (Fig. 4c). Expression of RAGE on brain microvessels was enhanced in Tg APPsw+/- mice (Fig. 4d left), and increased vascular expression of RAGE was associated with accumulation of Aß in AD brains (Fig 4d right).

Discussion

These data demonstrate that RAGE has an important role in Aß-mediated uptake at the BBB and its transport into the central

nervous system, as well as Aß-mediated cellular perturbation.

The first set of studies employed synthetic Aß infused in to wild-type mice, and the results apply to acute exposure of vasculature to Aß.

This invention provides the following methods:

A method for blockading RAGE, with either sRAGE or anti-RAGE IgG which thereby,

-suppresses binding to and uptake of Aß in relation to the vessel wall

-inhibits Aß-induced cell stress in the vasculature and in neurons, consequent to systemic infusion of Aß

Such an experimental model, although artificial, may be directly relevant to head trauma, stroke and other disorders in which there are acute elevations of Aß.

The second set of studies uses the Hsiao mice (reference for these is Hisao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G: correlative memory deficits, Aß elevation, and amyloid plaques in transgenic mice. Science 274:99, 1996). These experiments suggests that chronic exposure of vasculature to Aß results in RAGE-dependent vasoconstriction- thus, a RAGE blocker would be expected to increase cerebral blood flow in patients with increased levels of amyloid-beta peptide (at least when Aß is in the blood or blood vessel wall). These mice were made using the prion promoter, which expresses amyloid precursor protein in neurons and glial cells, predominately, but some seems to get into the vasculature as well. These mice are considered a

model of Alzheimer's disease. Thus, increasing cerebral blood flow in these mice could be interpreted as increasing cerebral blood flow in the setting of Alzheimer's disease. Decreased blood flow would be considered an adverse effect for cerebral function, thus, increasing blood flow would be considered (at least indirectly) neuroprotective.

The second set of studies actually is more powerful in terms of its implications since the mice are considered a model of Alzheimer's-type pathology.

Methods

Synthetic peptides: $A\beta_{1-40}$ and $A\beta_{1-42}$ human forms, and $A\beta_{1-40}$ murine form were be synthesized at the W M Keck Facility at Yale University using solid-phase tBOC(N-tert-butyloxycarbonyl)-chemistry, purified by HPLC, and the final products lyophilized and characterized by analytical reverse-phase HPLC, amino acid analysis, laser desorption mass spectrometry, as we previously described. [22,24] Stock solutions were prepared in DMSO to assure monomeric species, and kept at -80oC until use.

Radioiodination: of Aß was carried out with Na[^{125}I] and Iodobeads (Pierce), and the resulting components resolved by HPLC. [22,24]

Animals and tissue preparation: TgAPPsw+/- mice (bearing the double mutation Lys670Asn, Met671Leu) 9 months of age were in a mixed C57B6/SJL background, as were age-matched wild type control mice were used throughout the study. Animals were screened for the presence of the APP transgenes by PCR as described.[35] For histology, mice received intraperitoneal (i.p.) pentobarbital (150 mg/kg) and were perfused

transcardially with 0.1M PBS (pH 7.4) at 4°C. The right hemisphere was immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. The brain was cryoprotected in 30% sucrose in PBS at 4°C, and then fixed in paraformaldehyde as above at 4°C.

Cerebral blood flow measurement:. CBF was monitored by Laser Doppler Flowmetry (LDF, Transonic BLF21, NY) as we described. [36] LDF probes (0.8 mm diameter) were positioned on the cortical surface 2 mm posterior to the bregma, both 3 and 6 mm to each side of midline. The CBF was also determined by quantitative autoradiography using ^{14}C-iodoantipryine (IAP) using recently reported modified method in the whole mouse. [37] Briefly, 0.15 μCi ^{14}C-IAP was injected i.p. and animals sacrificed after 2 min. Blood from the frozen heart was sampled to obtain the final blood ^{14}C-IAP level. Frozen brains were coronally sectioned at 20 μm and exposed to autoradiographic film along with radioactive ^{14}C standards. After a 5 day exposure, the film was developed and the resulting images analyzed by quantitative autoradiography to determine levels of ^{14}C-IAP in individual brain regions. The CBF was calculated as reported: [37,40] $F = -\lambda \ln (1 - C_{IN\ (T)} / \lambda C_{PL})/T$, where F is the rate of flow per unit mass ($^{-1}$), $C_{IN\ (T)}$ is activity in unit weight of brain at time T, C_{PL} is the concentration of ^{14}C-IAP in the blood, and λ is the distribution ratio of ^{14}C-IAP between brain and the perfusion medium or blood at the steady state, i.e. 0.8.

Aß (4 nM/l) or vehicle were administered via femoral vein (n = 5 per group). α-RAGE, sRAGE etc.

Brain perfusion model. This model has bee extensively used to determine peptide and protein binding to and transport across the BBB. [22,23,38,39] For intra-arterial brain perfusion technique mice were anesthetized with i.p. ketamine (0.5

mg/kg) and xylazine (5 mg/kg), and the right common carotid artery isolated and connected to an extracorporeal perfusion circuit via fine polyethylene cannula (PE10). Details of the extracorporeal perfusion circuit were as reported elsewhere. [22,23,38,39] At the start of the perfusion, the contralateral common carotid artery was ligated, and both jugular veins severed to allow free drainage of the perfusate. Brains were perfused with oxygenated perfusion medium at a flow rate of 1 ml/min by peristalitic pump. The perfusion medium consisted of 20% sheep red blood cells (oxygen carrier) suspended in mock plasma containing 48 g/L dextran (FW 70 000) to maintain colloid osmotic pressure, and electrolytes and D-glucose (196 mg/dl) at concentrations corresponding to normal mouse plasma levels. Perfusion pressure and animal's own arterial blood pressure were continuously monitored. Blood gasses pO2, pCO2 and pH and electrolytes in the arterial inflow and in animal's own blood were monitored. All physiological parameters were kept within the normal range as we described. [22,23,38,39]

Injection of radioisotopes for transport studies. $[^{125}I]$-Aβ, ^{99m}Tc-albumin or ^{14}C-labeled inulin were infused into arterial inflow at a rate of 0.1 ml/min typically within 10 min for transport studies (corresponds to the linear phase of Aβ uptake). When the effects of different unlabeled molecular reagents were tested, those were injected 5 min prior to tracers injection and than simultaneously with radiolabled ligands. At predetermined times within 10 min mice were sacrificed by decapitation, and brain tissue prepared for radioactivity analysis. TCA and HPLC analysis as we described were used to determine molecular forms of uptake of radiolabeled Aß by the BBB. [22,23] Capillary-depletion technique was used to separate micravascular pellet from capillary-

depleted brain to quantify *in vivo* binding to microvessels vs. transport into brain parenchyma, as we reported.[22,23]

Mathematical modeling for transport studies. We have reported details of mathematical analysis elsewhere.[22,23,38,39] The uptake values for ^{125}I- Aß were based on the amount of intact molecule as determined by the TCA and HPLC analysis. The rate of entry (K_{IN}) is computed from eq. 1: $d[C_{IN\ (TEST\text{-}MOLEUCLE)} - C_{IN\ (ALBUMIN)}]/dt = K_{IN}\ C_{PL} - K_{OUT}\ [C_{IN\ (TEST\text{-}MOLECULE)} - C_{IN\ (ALBUMIN)}]$, where K_{OUT} is exit or efflux transfer coefficient, and R is the steady state or equilibrium ratio. Eq. 1 is integrated to give $[C_{IN\ (TEST\text{-}MOLECULE)} - C_{IN\ (ALBUMIN)}]/C_{PL} = R\ (1-e^{-KOUT\ T})$ (eq. 2). R is the steady state ratio, and the ratio K_{IN}/K_{OUT} at infinite time, and T is infusion time. Numerical values for K_{OUT} may be obtained from the slope of the plot of $\ln (R - [C_{IN\ (TEST\text{-}MOLECULE)} - C_{IN\ (ALBUMIN)}]/C_{PL})$ (eq. 3) against T, using the equation $K_{OUT} = -\ln(R - [C_{IN\ (TEST\text{-}MOLECULE)} - C_{IN\ (ALBUMIN)}]/C_{PL})/T$ (eq. 4). Finally, the value for K_{IN} is derived by substituting the number for K_{OUT} in: $K_{IN} = R\ K_{OUT}$ (eq. 5). When tracer uptake remains linear over studied period of time, the exist constant approaches zero, and $K_{IN} = d[C_{IN\ (TEST\text{-}MOLEUCLE)} - C_{IN\ (ALBUMIN)}]/dt\ C_{PL}$. The K_{IN} represents the fraction of circulating radioactive ligands that is taken up intact by 1 g of brain from 1 ml of plasma in 1 min, and is the same as the PS product if K_{IN} or PS << CBF,[39] a condition satisfied by Aß. Advanced graphics software and the MLAB mathematical modeling system (as above) will be used to obtain graphic plots and compute transfer coefficients.

Immunocytochemical analysis: for TNF-α, IL-6 and HO-1 in brains of wild type mice and TgAPPsw+/- mice was performed using standard techniques, as described (26). Briefly, fresh-frozen, acetone-fixed brain sections of wild type and

TgAPPsw+/- mice were stained with anti-TNF-a IgG (Santa Cruz), anti-IL-6 IgG (Santa Cruz and anti-HO-1 IgG (StressGen) as primary antibodies. The extent and intensity of staining in cellular elements was quantitated using the Universal Imaging System and NIH imaging systems. The relative intensity of cellular staining in control brain sections was compared to treated brains. Routine control sections included deletion of primary antibody, deletion of secondary antibody and the use of an irrelevant primary antibody.

Statistical analysis. Data from the proposed studies were analyzed by multifactorial analysis of variance (ANOVA) that ranged from one-way to three-way ANOVA. Each ANOVA included an analysis of residuals as a check on the required assumptions of normally distributed errors with constant variance. In the event the required assumptions were not satisfied, data transformations were considered. Appropriate multiple comparisons were included as a part of each analysis. For pair-wise comparisons, the Tukey method was used, and for comparisons with a control group we used Dunnett's test.

Example 2: RAGE at the Blood Brain Barrier Mediates Neurovascular Dysfunction Caused by Amyloid β_{1-40} peptide

Amyloid-beta peptides (Aβ) are important in the pathogenesis of Alzheimer's dementia. We found that the receptor for advanced glycation end products (RAGE) mediates *in vivo* transcytosis of circeulating Aβ_{1-40} across the blood-brain barrier (BBB) in mice. In an acute model in mice, blood to brain transport of Aβ_{1-40} (1-4 nM final plasma concentration) was coupled to its rapid neuronal uptake, cytokine responses

including enhanced production of tumor necrosis factor - α mRNA and protein and interleukin-6, neuronal oxidant stress (e.g. increased expression of hemoxygenase-1), and sustained reductions in cerebral blood flow (CBF). Aβ-induced cellular stress and cerebral vasospasm were blocked by circulating α-RAGE (40 μg/ml). In a chronic model, in 9-month old transgenic Tg APP sw +/- mice, CBF was significantly reduced by 63% in comparison to age-matched controls, this reduction was reversible by circulating α-RAGE in a dose-dependent fashion (10-40 μg/ml). In brains of subjects suffering from Alzheimer's disease, increased vascular expression of RAGE was associated with peri-vascular accumulation of Aβ, vascular and peri-vascular accumulation of proteins with nitrosylated amino-acid residues and increased expression of endothelial nitric oxide (NO) synthase. We conclude that vascular dysfunction caused by Aβ via RAGE at the BBB may contribute to ischemic changes and neurovascular injury in Alzheimer's dementia.

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